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Natural killer cell activities of synbiotic *Lactobacillus casei* ssp. *casei* in conjunction with dextran

T. Ogawa, Y. Asai, R. Tamai, Y. Makimura, H. Sakamoto, S. Hashikawa and K. Yasuda
Department of Oral Microbiology, Asahi University School of Dentistry, Gifu, Japan

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Correspondence: Dr Tomohiko Ogawa, Department of Oral Microbiology, Asahi University School of Dentistry, 1851–1 Hozumi, Mizuho, Gifu 501–0296, Japan.
E-mail: tomo527@dent.asahi-u.ac.jp

Summary

We have reported previously that *Lactobacillus casei* ssp. *casei*, together with specific substrate dextran, exhibited an adjuvant effect of stimulating humoral immune responses against bovine serum albumin (BSA) as a model antigen in BALB/c mice. In the present study, among the *Lactobacillus* species tested, *L. casei* ssp. *casei* with dextran significantly elevated the natural killer (NK) cell activites in spleen mononuclear cells from BALB/c mice in comparison to *L. casei* ssp. *casei* alone or other *Lactobacillus* species with or without dextran. Oral administration of *L. casei* ssp. *casei* together with dextran also resulted in a significant increase of NK cell activities in healthy human volunteers. Further, *L. casei* ssp. *casei* induced significant production of interleukin (IL)-12 in human peripheral blood mononuclear cells and IL-15 mRNA expression in the human intestinal epithelial cell line Caco-2. *L. casei* ssp. *casei* with dextran in food also significantly elevated the survival rate of BALB/c mice bearing Meth-A cells. Taken together, these results demonstrate that dietary synbiotic supplementation which is a combination of the *L. casei* ssp. *casei* used as a probiotic together with the dextran, a specific substrate as a prebiotic, efficiently elicits murine and human NK cell activities.

Keywords: cytokine, dextran, *Lactobacillus casei* ssp. *casei*, natural killer, synbiotic

Introduction

Potentiation of the intestinal mucosal barrier is thought to help protect from invasion by various pathogens. Lactic acid bacteria, Gram-positive and non-pathogenic organisms found in a wide variety of fermented food products [1], have been shown to provide health promoting and beneficial therapeutic effects toward the host [2,3], and are considered to function in a probiotic manner. Probiotic bacteria have been reported to prevent and treat inflammatory bowel disease in a number of previous studies [4]. They also protect infection by competing with pathogenic bacteria for attachment to gastrointestinal epithelium and enhance mucosal immune responses to pathogens [5].

Lactic acid bacteria have been found to have a variety of beneficial effects, including the prevention of carcinogenesis and tumour growth [6]. *Lactobacillus casei* and *L. acidophilus* were shown to inhibit the growth of transplantable tumour cells in experimental animals [7–9]. Biffi *et al.* also demonstrated that milk fermented with *L. acidophilus* and *L. paracasei* reduced the growth of a human breast cancer cell line MCF7 *in vitro* [10]. In addition, up-regulation of natural killer (NK) cell activity was observed in mice administered orally with *L. casei* in mice [11].

The immunoregulatory cytokine interleukin (IL)-12, a 70-kDa heterodimer formed by the covalent assembly of two chains, 40 kDa and 35 kDa, mediates interferon (IFN)-γ production from T cells and NK cells, and augments their cytotoxic activity against tumour cells [12]. Therefore, IL-12-activating capacity seems to be associated with anti-tumour activity. Interleukin (IL)-15 has a 4-helix bundle structure and exhibits IL-2-like functions, such as the induction of T cell and NK cell proliferation [13]. IL-15 is able to induce bone marrow-derived CD34-positive haematopoietic progenitor cells to differentiate into CD3-negative- and CD56-positive-NK cells [14] and up-regulates NK cell cytolytic functions [15]. Further, IL-15 mRNA is expressed by non-T cells, including those found in kidney, placenta and skeletal muscle tissue, as well as by macrophages and epithelial cells, unlike IL-2, which is induced only by T cells [16]. It was also

reported that intestinal epithelial cells expressed IL-15 mRNA, which was up-regulated by interferon (IFN)-γ [17].

A synbiotic is a combination of live bacteria used as a probiotic and the specific substrate used as a prebiotic, which is defined as non-digestible food ingredients that affect the host beneficially by selective stimulation of some bacterial species in the colon [18]. It has been proposed that enhancement of probiotic bacteria in the intestines provides advantages to the host [19]. It was also demonstrated that synbiotic nutritional supplements consisting of probiotic *L. paracasei* and prebiotic fructooligosaccharides efficiently increased NK cell activity in elderly people [20]. Recently, we have shown that *L. casei* ssp. *casei* has a specific ability to metabolize macromolecular dextran, as oral administration of the bacteria in conjunction with dextran effectively enhanced humoral immune responses to BSA in BALB/c mice [21]. In the present study, we evaluated NK cell activities of *L. casei* ssp. *casei* administered together with dextran.

Materials and methods

Bacterial strains

L. casei ssp. *casei* JCM 1134^T, *L. paracasei* ssp. *paracasei* JCM 1053 and *L. acidophilus* JCM 1132^T were obtained from the Japan Collection of Microorganisms (Riken Biosource Center, Saitama, Japan).

Growth experiments

Growth experiments with the *Lactobacillus* species were carried out at 37°C by measuring optical density at 660 nm (OD_{660}) with a colorimeter (Digital Bench Colorimeter Model 21150; Industrial & Chemical Measurement, Hillsboro, OR, USA). Fifty microlitres of each bacterial suspension (adjusted to 0·5 McFarland standard) was inoculated into 5 ml of deMan Rogosa Sharpe (MRS) broth (BD Biosciences, Mountain View, CA, USA) supplemented with or without 2% dextran, which has a molecular weight of 10 000 (Serva, Heidelberg, Germany), after which bacterial cell growth was monitored every 12 h. During the measurement of OD_{660}, a non-inoculated culture medium was used as a blank.

Preparation of murine mononuclear cells

Eight-week-old male BALB/c mice (Charles River Japan, Inc., Yokohama, Japan), which received pellet chow (MF diet, Oriental Yeast Industry Co., Tokyo, Japan), were divided into two groups (each containing six mice). On days 0–9, the control group was given MF powdered chow (control group) and the dextran group received MF powdered chow with 75 mg/kg of dextran supplement. Mice in both groups received oral administrations with or without 1×10^7 CFU of various *Lactobacillus* species by gastric intubation with the aid of an intubation needle on days 0, 1 and 2. On day 9, murine mononuclear cells (MNC) as effector cells for a ^{51}Cr-release assay were isolated from the spleen cells using Histopaque®1082 (Sigma, St Louis, MO, USA) separation.

Preparation of human peripheral blood mononuclear cells

This experiment was performed with eight healthy adult volunteers (six males, two females; average age 34·9 years). All subjects were informed regarding the study and each signed an informed consent form approved by the Ethics Committee of Asahi University (reference number 15007). On days 1–7, each received 1×10^{10} CFU of lyophilized *L. casei* ssp. *casei* and 1 g of dextran once per day. On days 0, 8 and 11, heparinized venous blood was sampled and subjected to fractionation using a Histopaque®1077 (Sigma) to obtain human peripheral blood mononuclear cells (PBMC) as effector cells for a ^{51}Cr-release assay.

Cell lines

NK-sensitive YAC-1 (mouse T cell leukaemia) and K562 (human erythroleukaemia) cell lines were used as target cells for ^{51}Cr-release assays, after being maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO_2. The culture medium was replaced every 3 days with fresh medium.

The human intestinal epithelial cell line Caco-2 was maintained in Eagle's minimum essential medium (EMEM; Sigma) supplemented with 10% FBS (Sigma) and 0·1 mM of non-essential amino acids (Gibco Laboratories, Grand Island, NY, USA). Meth A fibrosarcoma of BALB/c mouse origin was maintained by serial intraperitoneal passages in syngeneic BALB/c mice. The cells were prepared just prior to the experiment with mice peritoneal cavity specimens.

^{51}Cr release assay

A standard 4-h ^{51}Cr release assay was used to determine necrotic death of the target cells. Briefly, 1×10^6 target cells were labelled with 50 μCi of $Na_2{}^{51}CrO_4$ for 1 h at 37°C. Then, the cells were washed twice and resuspended in RPMI-1640 supplemented with 10% FBS, and YAC-1 (2×10^4 cells) or K562 (1×10^4 cells) cells were aliquoted in wells in volumes of 100 μl. Effector cells in an equal volume were also added six times to give the desired effector : target (E : T) cell ratios. The plates were incubated at 37°C for 4 h and then centrifuged at 370 ***g*** for 10 min. Next, the culture supernatants were decanted and samples were counted using a counter (Auto Well Gamma System ARC-380 CL; Aloka Co., Ltd, Tokyo, Japan). Medium alone or 1% Nonidet P-40 (Sigma)

was added to labelled target cells to determine spontaneous (spons) and maximum (max) release, respectively. The percentage of killed cells (% kill) was calculated using the following equation: % kill = (experimental – spons)/(max – spons) × 100%. Before measurement of the cohort members, we examined different E : T ratios (1%, 3%, 11%, 33% and 100%) in a pilot study with a small amount of cytotoxic activity. We chose the ratio of 33%, which was the ratio at which differences in cytotoxic activity between individuals were most distinguishable.

IL-12 production

Human PBMC were isolated from heparinized venous blood sampled from a healthy adult donor as described above, then washed three times with phosphate buffered saline (PBS) (Sigma) and incubated with the indicated cells of *Lactobacillus* species in RPMI-1640 medium with 10% FBS at 37°C for 48 h. Prior to the experiment, we confirmed the ability of *Lactobacillus* species to survive in this culture condition. Culture supernatants were collected and analysed by enzyme-linked immunosorbent assay (ELISA) for secreted IL-12 (*e*Bioscience, San Diego, CA, USA). The results were determined using a standard curve prepared for each assay.

IL-15 mRNA expression

Caco-2 cells were co-cultured with 1×10^6 CFU/ml of *Lactobacillus* species or stimulated with 100 ng/ml of human IFN-γ (*e*Bioscience) in EMEM with 10% FBS at 37°C for 4 h. Total cellular RNA from the cells was extracted using an RNAqueous-4PCR (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Extracted RNA (1 μg) was then reverse-transcribed into first-strand cDNA at 42°C for 40 min according to the manufacturer's instructions. PCR amplification was performed using the following oligonucleotide specific primers: for β-actin, sense 5′-GTG GGG CGC CCC AGG CAC CA-3′ and anti-sense 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′), and for human IL-15, sense 5′-GGA TTT ACC GTG GCT TTG AGT A-3′ and anti-sense 5′-TTC CTC CAG TTC CTC ACA TTC T-3′. Next, 5 μg of cDNA from the samples was amplified with 0·2 μM of the sense and anti-sense primers for the target gene in a 50-μl reaction mixture containing 75 U/ml of Ex *Taq* polymerase (Takara Biochemicals, Shiga, Japan). After initial denaturation at 94°C for 2 min, 35 cycles of denaturation (94°C for 30 s), annealing (60°C for 1 min) and extension (72°C for 1 min) for IL-15, and 27 cycles of denaturation (94°C for 30 s), annealing (58°C for 1 min) and extension (72°C for 1 min) for β-actin were performed using a PCR Express Thermal Cycler (Hybaid, Middlesex, UK). As a negative control, a non-RT sample was amplified by PCR. Following PCR, 10 μl of the total amplified product was electrophoresed on an ethidium bromide-stained 1% agarose gel and fluorescence was visualized under UV light.

NK cell fraction in human PBMC

Human PBMC were labelled for analysis with fluorescein isothiocianate (FITC)-conjugated anti-human CD3 (clone UCHT1 +) and R-phycoerythrin (RPE)-conjugated anti-human CD56 (clone MOC-1) antibodies (Dako Dual-Colour Reagent, Dako, Glostrup, Denmark). The cells were then washed with PBS and fixed with 1% paraformaldehyde. The percentage of CD56-positive/CD3-negative NK cells in the lymphocyte fraction was analysed using a FACS Calibur with Cell Quest software (BD Biosciences, San Jose, CA, USA) after gating on the forward- and side-scatter profile.

Anti-tumour activity against tumour-bearing mice

The BALB/c mice that received the MF diet were divided into four groups (each containing 12 mice). Starting from 7 days before inoculation with tumour cells, the mice were given MF powdered chow (control group), MF powdered chow with 75 mg/kg of dextran supplement (dextran group), 5×10^5 CFU/day of lyophilized *L. casei* ssp. *casei* in MF powdered chow (*L. casei* group) or 5×10^5 CFU/day of lyophilized *L. casei* ssp. *casei* in MF powdered chow with 75 mg/kg of dextran supplement (*L. casei*–dextran group), and then inoculated intraperitoneally with Meth A cells at a dose of 1×10^4 cells/mouse. Survival was monitored for up to 80 days.

Statistical analysis

The normality of NK cell activities was confirmed by χ^2 for goodness of fit, and the statistical significance of NK cell activities in mice and humans was quantified using unpaired and paired *t*-tests, respectively. Survivals were calculated starting from the day of inoculation of Meth A cells. Survival curves were drawn according to the Kaplan–Meier method, with differences analysed by a log-rank test. The significance level was set at 5%.

Results

Cell growth of *Lactobacillus* species in the presence of dextran

To evaluate the ability of dextran to be utilized with *L. casei* ssp. *casei*, *L. paracasei* ssp. *paracasei* and *L. acidophilus*, we monitored cell growth by measuring OD_{660}. Increased cell growth with the addition of dextran was observed in the case of *L. casei* ssp. *casei* (Fig. 1). In contrast, *L. paracasei* ssp. *paracasei* and *L. acidophilus* showed almost identical cell growth patterns, regardless of the presence or absence of dextran.

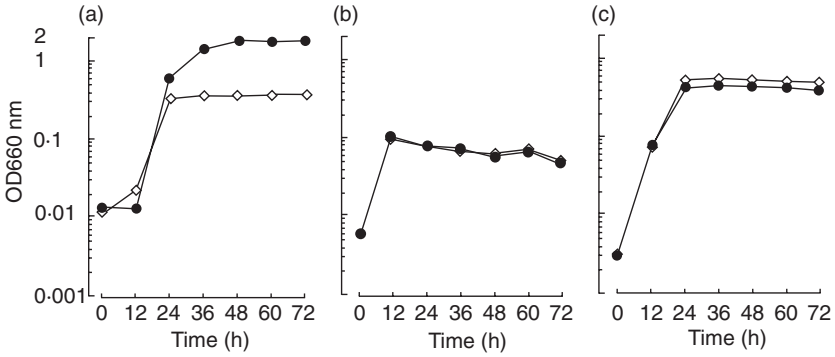

Fig. 1. Cell growth of *Lactobacillus* species in the presence or absence of dextran. Fifty microlitres of a bacterial suspension of *Lactobacillus casei* ssp. *casei* (a), *L. paracasei* ssp. *paracasei* (b) or *L. acidophilus* (c) (adjusted to 0·5 McFarland standard) was inoculated onto 5 ml of deMan Rogosa Sharpe (MRS) broth supplemented with (closed circle) or without (open diamond) 2% dextran, and then incubated aerobically at 37°C. Bacterial cell growth was monitored by measuring optical density at 660 nm (OD_{660}) with a colorimeter every 12 h. During the measurement of OD_{660}, a non-inoculated culture medium was used as a blank. Experiments were done at least three times and representative results are presented.

Effects of *L. casei* ssp. *casei* on NK cell activities in dextran-fed BALB/c mice

To examine the effects of *Lactobacillus* species with or without dextran toward host cells, we investigated NK cell activities in BALB/c mice administered orally with *Lactobacillus* species (Fig. 2). All the bacteria tested significantly increased the level of NK cell activity in non-dextran-fed mice. Among them, oral administration of *L. casei* ssp. *casei*, but not *L. paracasei* ssp. *paracasei* or *L. acidophilus*, resulted in a significant increase of NK cell activity in dextran-fed mice compared with non-dextran-fed mice. These results suggest that the specific utilizing ability of dextran by *L. casei* ssp. *casei* contributes to an up-regulation of NK cell activity.

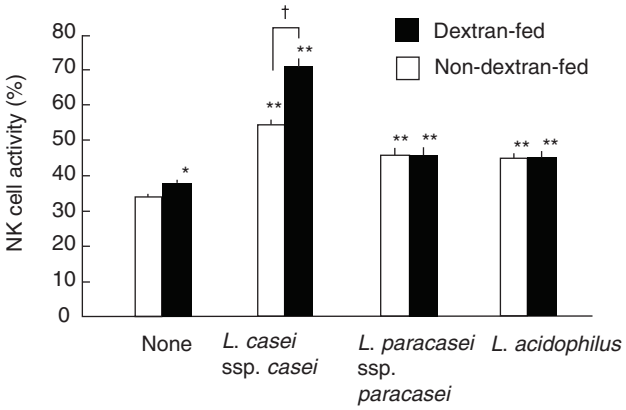

Fig. 2. Effects of *Lactobacillus casei* ssp. *casei* on natural killer (NK) cell activities in dextran-fed mice. BALB/c mice received MF powdered chow (non-dextran-fed) or MF powdered chow with 75 mg/kg of dextran supplement (dextran-fed), and then were administered orally with or without 10^7 colony-forming units (CFU) of various *Lactobacillus* species by gastric intubation on days 0, 1 and 2. On day 9, mononuclear cells (MNC) were isolated from spleen cells and NK cell activities were measured as described in Materials and methods. The mean values were significantly different from non-administration in non-dextran-fed mice (**$P < 0·01$, *$P < 0·05$) and administration of *L. casei* ssp. *casei* in non-dextran-fed mice (†$P < 0·01$).

IL-12 production by human PBMC co-cultured with *L. casei* ssp. *casei*

It has been reported previously that administration of *Lactobacillus* species killed by UV radiation induced IL-12 production by human PBMC [22]. In the present study, we examined the IL-12-producing activity in human PBMC co-cultured with *Lactobacillus* species. We found that *L. casei* ssp. *casei* induced greater IL-12 production in a cell number-dependent manner compared with *L. paracasei* ssp. *paracasei* and *L. acidophilus* (Fig. 3).

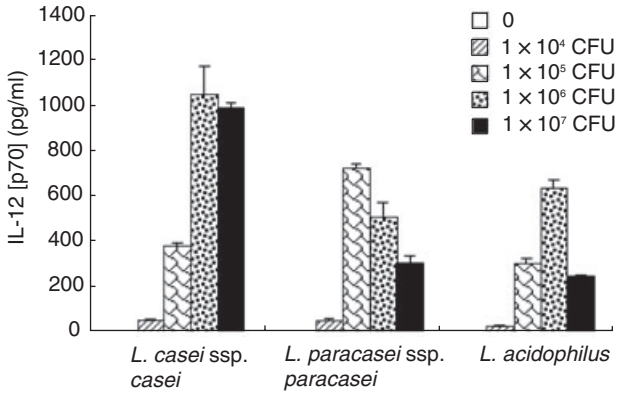

Fig. 3. Interleukin (IL)-12 production by human peripheral blood mononuclear cells (PBMC) co-cultured with *Lactobacillus casei* ssp. *casei*. The cells were co-cultured with the indicated doses of live *L. casei* ssp. *casei*, *L. paracasei* ssp. *paracasei* or *L. acidophilus* for 48 h. IL-12 production was analysed by enzyme-linked immunosorbent assay (ELISA). Data are shown as the mean ± s.e.m. of three independent experiments.

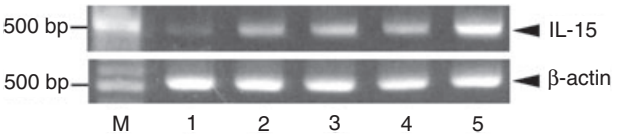

Fig. 4. Interleukin (IL)-15 mRNA expression in Caco-2 cells co-cultured with *Lactobacillus casei* ssp. *casei*. The cells were co-cultured with 1×10^6 colony-forming units (CFU)/ml of *Lactobacillus* species for 4 h. Interferon (IFN)-γ (100 ng/ml) was used as a positive control stimulant. IL-15 mRNA expression was analysed by reverse transcription-polymerase chain reaction (RT-PCR), with β-actin assayed as a positive control. Lanes: M, 100 base pairs (bp) ladder marker; 1, Caco-2 cell alone; 2, *L. casei* ssp. *casei*; 3, *L. paracasei* ssp. *paracasei*; 4, *L. acidophilus*; and 5, IFN-γ.

Expression of IL-15 mRNA in human intestinal epithelial cells co-cultured with *L. casei* ssp. *casei*

Because intestinal epithelial cells have been reported to express IL-15 [17], we examined IL-15 mRNA expression in Caco-2 cells co-cultured with *Lactobacillus* species by reverse transcription-polymerase chain reaction (RT-PCR). Caco-2 cells constitutively expressed IL-15 mRNA, and all the tested bacteria as well as IFN-γ clearly induced the expression (Fig. 4). RT-PCR analysis of β-actin expression confirmed the quality of all RNA preparations and no band was detected for the non-RT sample by PCR (data not shown).

Effects of feeding of *L. casei* ssp. *casei* with dextran on NK cell activities in humans

To investigate the effects of oral *L. casei* ssp. *casei* together with dextran, we examined NK cell activities in eight healthy human volunteers (Fig. 5). The average NK cell activity was 28·7 ± 9·8% (range 12·7–45·3%) on 1 day before supplementation, which increased to 43·9 ± 15·9% (range 19·9–64·1%) on day 8, 1 day after finishing supplementation for 7 days. A significant increase was also observed on day 11, 4 days after finishing the supplementation period (41·7 ± 13·2%; range 17·9–62·0%). Interestingly, the percentage of NK cells in the peripheral blood lymphocyte fractions showed a tendency to continue increasing after supplementation (data not shown). These results suggest that orally ingested *L. casei* ssp. *casei* together with dextran facilitates the number and ability of NK cells in human.

Anti-tumour activities of *L. casei* ssp. *casei* with dextran

Figure 6 shows that the results for BALB/c mice that survived the trials using autologous Meth A cells. The survival rate of the control group was 8·3%, whereas that of the dextran and *L. casei* groups was 33·3%, respectively. Furthermore, the *L. casei*–dextran group showed a significant increase in survival rate (50%) compared to the others. These results indicated that feeding of *L. casei* ssp. *casei* together with dextran markedly elevated anti-tumour activities.

Discussion

We have demonstrated previously that *L. casei* ssp. *casei* is specifically capable of utilizing dextran [21]. It has been also shown that oral administration of the bacteria in conjunction with dextran effectively augments humoral immune responses in mice. In the initial experiment of this study, we investigated the effects of dextran on bacterial cell growth of three *Lactobacillus* species using a colorimeter (OD_{660}). Those results showed that *L. casei* ssp. *casei* could utilize dextran and additional growth was seen, compared with in the absence of dextran (Fig. 1). Therefore, we concluded that dextran supplementation plays an important role in the further growth of *L. casei* ssp. *casei*.

It has been demonstrated previously that an intravenous injection of *L. casei* LC 9018 augmented the NK cell activities of spleen cells in BALB/c mice [23]. Takagi *et al*. also showed that oral administration of *L. casei* Shirota enhanced NK cell activity and caused a delay of 3-methylcholanthrene-induced carcinogenesis in mice [24]. In the present experiments, we found that oral administration of *L. casei* ssp. *casei* JCM 1134^T augmented NK cell activities in BALB/c mice, while dextran supplementation resulted in further increases of only the activities of *L. casei* ssp. *casei* (Fig. 2). These results indicate that the dextran-utilizing ability of *L. casei*

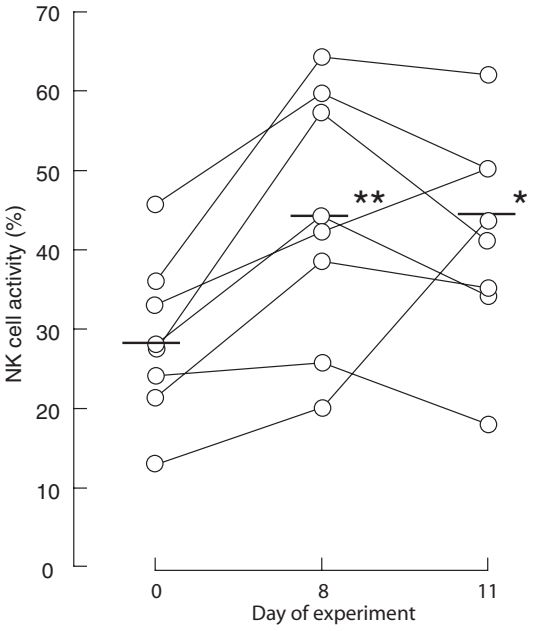

Fig. 5. Effects of orally ingested *Lactobacillus casei* ssp. *casei* together with dextran on human natural killer (NK) cell activities. Eight healthy adult volunteers received 1×10^{10} colony-forming units (CFU) of lyophilized *L. casei* ssp. *casei* and 1 g of dextran once per day from days 1–7. On days 0, 8 and 11, peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood and NK cell activities were measured as described in Materials and methods. The mean values were significantly different from experimental day 0 (**$P < 0{\cdot}01$, *$P < 0{\cdot}05$).

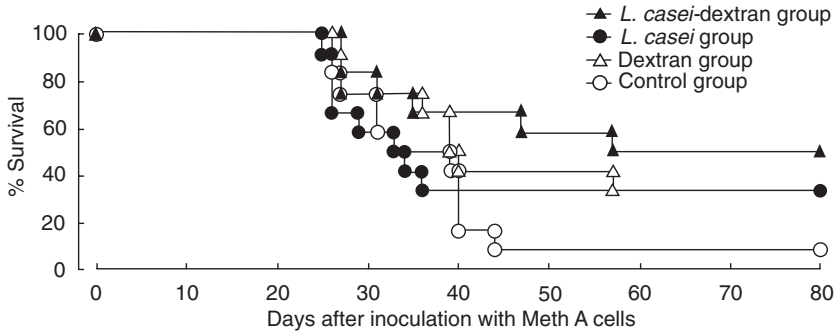

Fig. 6. Effects of feeding *Lactobacillus casei* ssp. *casei* together with dextran on the survival rate of Meth A-bearing mice. BALB/c mice received MF powdered chow (control group), MF powdered chow with dextran (dextran group), lyophilized *L. casei* ssp. *casei* in MF powdered chow (*L. casei* group) or lyophilized *L. casei* ssp. *casei* in MF powdered chow with dextran (*L. casei*–dextran group), and then were inoculated intraperitoneally with Meth A cells at a dose of 1×10^4 cells/mouse. A *P*-value of 0·029 was determined using a Kaplan–Meier product limited-survival analysis between the *L. casei*–dextran and control groups.

ssp. *casei* contributes to further augmentation of NK cell activity (Figs 1 and 2).

Little is known about the mechanisms of increased NK cell activity by oral administration of *Lactobacillus* species. It was reported that *L. plantarum*, *L. rhamnosus* and *L. paracasei* ssp. *paracasei* activated human PBMC to induce IL-12 [22,25]. The major biological activities of IL-12 are directed toward T cells and NK cells, in which it increases cytokine production, proliferation and cytotoxicity [26]. Thus, IL-12 production is considered to be related closely to NK cell activity. Our data also showed that *L. casei* ssp. *casei* clearly induced IL-12 production by human PBMC (Fig. 3). In addition, we demonstrated that *L. casei* ssp. *casei* as well as the other *Lactobacillus* species used in this study induced IL-15 mRNA expression in the human intestinal epithelial cell line Caco-2 (Fig. 4). Reinecker *et al*. also reported that IL-15 mediated the proliferation of Caco-2 cells [17], while IL-15 induced by intestinal epithelial cells was shown to mediate the activation of intestinal intraepithelial NK cells [27]. Together, these results suggest that the cytokines induced by *L. casei* ssp. *casei* are related closely to the activation of NK cells.

Dietary probiotic supplementation, such as food fermented with lactic acid bacteria, has been demonstrated to have health-promoting effects through improvement of the intestinal microflora and host immune system [3]. It was demonstrated that daily intake of fermented milk containing *L. casei* DN114001 for 8 weeks enhanced the innate immune defence in healthy middle-aged humans [28]. Gill *et al*. also showed that dietary consumption of milk supplemented with *L. rhamnosus* and *Bifidobacterium lactis* for 3 weeks enhanced NK cell activity in elderly people [29]. Furthermore, oral supplementation of a synbiotic diet containing fructooligosaccharides, along with *L. acidophilus* and *Bifidobacterium* species, was reported to dramatically facilitate weight gain in acutely ill children receiving antibiotics [30]. Femia *et al*. also demonstrated that the prebiotic inulin in conjunction with probiotics *L. rhamnosus* and *B. lactis* efficiently exerted protective effects on azoxymethane-induced colon cancer in rats [31]. In the present study, oral administration of *L. casei* ssp. *casei* together with dextran resulted in a further increase of NK cell activities in humans and mice (Figs 2 and 5), as well as of the percentage of NK cells in the lymphocyte fraction in healthy human volunteers (data not shown). Furthermore, Meth A-bearing BALB/c mice, which received *L. casei* ssp. *casei* together with dextran, had a greatly increased survival rate compared with the control group (Fig. 6). Together, these results suggest that oral supplementation of synbiotic materials enhance host immune functions and augment anti-tumour activities.

In conclusion, our results indicate clearly that a new synbiotic supplement consisting of the probiotic *L. casei* ssp. *casei* and the prebiotic dextran would be highly efficacious when given as an oral immunoadjuvant. Moreover, dextran, which is utilized specifically by *L. casei* ssp. *casei*, appears to play a pivotal role as both a selective prebiotic material and adjuvant.

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